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13. Abstract *(Maximum 200 Words)* *(abstract should contain no proprietary or confidential information)*

Insulin-like growth factor-1 (IGF-I) stimulates proliferation of MCF-7 cells via the type I IGF receptor (IGF1R) and causes phosphorylation of the adaptor protein, insulin receptor substrate-1 (IRS-1). Interleukin 4 (IL-4) inhibits breast cancer cells and also phosphorylates IRS-1. The hypothesis is that IGF-I and IL-4 phosphorylate different residues with IGF-I treatment targeting IRS-1 for degradation via the proteasome. The goal of this project is to identify amino acids of IRS-1 phosphorylated by IGF-I compared to IL-4 using two-dimensional electrophoresis (2DE) of phosphorylated IRS-1. Several technical difficulties and limitations of 2DE have been encountered and impeded the completion of the tasks. Unfortunately, it appears that the large size, abundance in cells, and perhaps charge of IRS-1 may make it difficult to use this technique. Despite sustained efforts, it has not been possible to detect IRS-1 after 2DE. As the long-term objective of my training is to identify new targets for breast cancer therapy, I have also worked on another project to inhibit IGF-I action in breast cancer cells using a humanized single chain antibody against IGF1R. In the coming year, work will be continued on 2DE of IRS-1 along with an alternate approach of mutating residues in the putative destruction box motif of IRS-1 in an attempt to inhibit the mitogenic effects of IGF-I.

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ANNUAL SUMMARY REPORT

INTRODUCTION:

Insulin like growth factor-1 (IGF-1) is a potent mitogen for breast cancer cells and the IGF system is a key growth regulatory pathway in breast cancer. Binding of IGF-1 to the type I IGF receptor (IGFIR) stimulates a cascade of signaling events leading to cell growth. IGF-1 transduces its signal through the adaptor protein insulin receptor substrate-1 (IRS-1). IGF-1 treatment results in phosphorylation of IRS-1. The cytokine interleukin-4 (IL-4) inhibits breast cancer cells. Our laboratory has shown that IL-4 acting via its receptor stimulates programmed cell death and also phosphorylates IRS-1. Thus, two factors with opposing effects on breast cancer cells cause phosphorylation of IRS-1. We have shown that IGF-1 causes a higher level of phosphorylation of IRS-1 than IL-4. In addition, IGF-1 causes acute phosphorylation of IRS-1 with rapid loss of detectable IRS-1 while IL-4 causes sustained phosphorylation of IRS-1. Phosphorylation of IRS-1 by IGF-1 causes degradation of IRS-1 by the ubiquitin proteasome pathway.

My post-doctoral project proposed to study the mechanisms by which IRS-1 is coupled with growth stimulatory pathways with the aim of answering the question: How can phosphorylation of IRS-1 be involved in both cell growth and cell death?
The hypothesis was that IRS-1 is differentially phosphorylated by IGF-1 and IL-4 in breast cancer cells and that the phosphorylation of IRS-1 by IGF-1 results in phosphorylation of specific serine/threonine residues which targets IRS-1 for degradation via the ubiquitin pathway. This hypothesis is to be tested by identifying the tyrosine, serine and threonine residues of IRS-1 phosphorylated by IGF-1 and IL-4 using two-dimensional gel electrophoretic separation of phosphorylated IRS-1 followed by phosphoaminoacid analysis and sequencing of phosphopeptides that differ between IGF-1 and IL-4 stimulation of IRS-1. Thus, the goal of this project is to identify those residues of IRS-1, which are phosphorylated by IGF-1 and not IL-4 and are, thus, responsible for targeting IRS-1 for degradation and then make a mutant IRS-1 that can neutralize the action of IGF-1 or may be change a mitogenic signal to a death signal.

BODY:

As I had mentioned in my annual summary report of June 2001, several technical difficulties were encountered during two-dimensional SDS-polyacrylamide gel electrophoresis (2-D SDS-PAGE) of IRS-1 and the tasks listed in the Statement of Work have not been accomplished. It is recently becoming evident that due to the limitations of 2-D SDS-PAGE (1), it may not be possible to successfully use 2DE for large proteins such as IRS-1, which is a 185 kDa protein.

As stated in my annual summary report of June 2001, 2D-E of IRS-1 was performed using:

-First dimension isoelectric focusing (IEF) with 11 cm immobilized IPG strips of pH 3-10 (Amersham Biosciences, Piscataway, NJ).

-Second dimension SDS-PAGE using 8% Tris-Glycine gels.

Despite several attempts I was unable to detect IRS-1 by immunoblotting after 2DE.

A major portion of this year was also spent in optimizing 2DE for large proteins. 2DE was performed using IPG strips of various lengths. Both the 13 cm and 18 cm strips were used but I was unable to detect IRS-1. For the second dimension SDS-PAGE 8-16% Tris-glycine gradient gels (Bio-Rad, Hercules, CA) were used. It has been reported that ZOOM gels (Invitrogen Life Technologies) which are specifically designed for 2DE give better resolution and separation for 2DE. However, IRS-1 was not detected after 2DE using either the 8-16 % gradient gels or ZOOM gels.

Various solubilization and reducing agents such as thiourea, tributyl phosphine, and dithioerythritol were tried in order to reduce the protein and maximize its solubility. To eliminate the possibility that poor solubility of IRS-1 in the cell lysate in the various buffers tried was responsible for these problems, we also used a protein extraction kit, the ReadyPrep sequential extraction kit (Bio-Rad, Hercules, CA), to prepare cellular proteins for 2DE. This reagent is specifically sold to extract proteins of differing solubility from cell lysates in a form that is suitable for 2DE. IRS-1 was not detected in cell lysates prepared with this kit either. Increasing concentration of urea and addition of thiourea also did not help. All reagents such as urea, thiourea, DTT, and bromophenol blue used were of the highest purity for 2D-E to eliminate solubility problems caused by impurities in reagents. As shown in Figure 2, use of urea and thiourea in the rehydration buffers increased 2D spots of high molecular weight proteins that were obtained as visualized by silver staining of the gel after 2DE. However, no IRS-1 was detected after immunoblotting with an antibody against IRS-1 as shown in Figure 3. In contrast, I was able to detect IGF1R by immunoblotting after 2DE. As shown in Figure 4, the β-subunit of IGF1R was readily detected by immunoblotting after 60 μg of MCF-7 cell lysate was subjected to 2DE. The β-subunit of IGF1R is a 97 kDa protein. Figure 4 A shows 2DE of MCF-7 cells without IGF-I treatment and 4 B with IGF-I treatment. In both cases, IGF1R was readily detected. In Figure 4 B, IGF1R in MCF-7 cell lysates following only SDS-PAGE is shown to the right of the IPG strip to show that the spot seen on 2DE is the same size as the band after SDS-PAGE.

As an alternate approach to determine the residues or motifs that may target IRS-1 for degradation, two hemagluttinin A (HA) tagged mutants of human IRS-1 have been obtained from Dr. Adrian Lee (Baylor College of Medicine, Houston, TX). These mutants delete varying regions of IRS-1 from the C-terminal end. Stable cells lines expressing these deletion mutants of IRS-1 are being created and will be tested for their effect on the degradation of IRS-1 by the proteasome and growth of breast cancer cells. In addition, human IRS-1 has a putative destruction box signal at residues 1074-1082 which may target it for degradation by the proteasome. I am mutating residues in the destruction box signal of IRS-1 to determine if inhibition of degradation occurs and if degradation of IRS-1 is linked to mitogenesis in MCF-7 cells.

In addition to the experiments outlined in the fellowship training grant I am working on another project with the goal of inhibiting IGF action in breast cancer cells. We have used a humanized single chain antibody against the type I IGF1R to inhibit breast cancer

cells. We have submitted a manuscript describing the mechanism of this antibody in May of this year (see bullet list of Reportable Outcomes). I am currently studying the effects of this antibody *in vivo* using an MCF-7 xenograft model of breast cancer. In the past year, I have also worked on a third project investigating the role of IGF1R in breast cancer cell proliferation, motility and metastasis. I have used a C-terminally truncated IGF1R which behaves in a dominant negative manner. This truncated receptor was stably transfected into a metastatic variant of MDA-MB-435 cells, LCC6 cells (2) and the effect of the truncated dominant negative IGF1R on breast cancer proliferation and metastasis has been investigated. I am currently preparing a manuscript on this work on the dominant negative receptor.

KEY RESEARCH ACCOMPLISHMENTS:

- Optimization of technical aspects of 2D electrophoresis for large proteins

- Characterization of the *in vitro* effects of a single-chain antibody against the IGF1R and the mechanism of action by which it renders breast cancer cells refractory to the mitogenic effects of IGF-I.

REPORTABLE OUTCOMES:

- Submitted a manuscript to *Cancer Research* in May 2002.
  Deepali Sachdev, Shu-Lian Li, Yoko Fujita-Yamaguchi, Jeffrey S. Miller and Douglas Yee. A chimeric humanized single chain antibody against the type I insulin-like growth factor receptor renders breast cancer cells refractory to the mitogenic effects of insulin-like growth factor-I.

- Abstract submitted for the 93rd Annual meeting of the American Association for Cancer Research was selected for an oral presentation in a minisymposium titled "Experimental/ Molecular Therapeutics: Growth Factor Receptors/Surface Antigens as Therapeutic Targets" on April 10, 2002.
  Sachdev, D, Fujita-Yamaguchi, Y, and Yee, D. A single chain antibody against the type I insulin-like growth factor receptor makes breast cancer cells refractory to the mitogenic effects of IGF-I (see appendix).

- Invited as a speaker at a symposium on "Recent Advances in the Pharmacology of Breast Cancer" at the Experimental Biology 2002 meeting in New Orleans, LA. I gave a 25-minute oral presentation titled "The insulin-like growth factor system as a target for therapy of breast cancer". This symposium was part of the American Society for Pharmacology and Experimental Therapeutics meeting.

- Submitted an abstract for the San Antonio Breast Cancer Symposium to be held in San Antonio in December 2002.

CONCLUSIONS:

Due to various technical difficulties and limitations of two-dimensional electrophoresis technology, the proposed statement of work has not been accomplished. In the coming year I will continue to work on 2DE of IRS-1. I will also make IRS-1 mutants that alter the destruction box signal present at residues 1074-1082 of human IRS-1 to determine if prevention of degradation by the proteasomes can affect the mitogenic response of breast cancer cells to IGF-I. The long-term goal of my project is to identify suitable targets for treatment of breast cancer. Towards that end, I am also working on a single chain antibody against IGF1R that we have shown can make MCF-7 cells refractory to IGF-I.

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1. Jenkins, R. E. and Pennington, S. R. 2001. Arrays for protein expression profiling: towards a viable alternative to two-dimensional gel electrophoresis?, Proteomics. *1:* 13-29.

2. Leonessa, F., Green, D., Licht, T., Wright, A., Wingate-Legette, K., Lippman, J., Gottesman, M. M., and Clarke, R. 1996. MDA435/LCC6 and MDA435/LCC6MDR1: ascites models of human breast cancer, Br J Cancer. *73:* 154-61.

APPENDIX:

Figure 1: Copy of the abstract presented at the Annual Meeting of the American Society for Cancer Research in April 2002.

#4983 A humanized single chain antibody against the type I insulin-like growth factor receptor makes breast cancer cells refractory to the mitogenic effects of IGF-I. Deepali Sachdev, Yoko Fujita-Yamaguchi, and Douglas Yee. *University of Minnesota Cancer Center, Minneapolis, MN, and Tokai University, Hiratsuka, Japan.*

Insulin-like growth factors (IGFs) are potent mitogens for breast cancer cells stimulating their proliferation, motility and survival. The type I IGF receptor (IGF1R) is a transmembrane receptor tyrosine kinase that mediates the effects of IGF-I. In MCF-7 cells, we have shown that IGF-I activates IGF1R, which phosphorylates the adaptor protein, insulin receptor substrate-1 (IRS-1). IRS-1 then recruits other signaling molecules and this results in the activation of further downstream pathways including the mitogen-activated protein kinase (MAPK) and phosphatidylinositol 3'-kinase (PI3K) pathways. Thus, inhibition of IGF1R activation should inhibit IGF action in breast cancer cells. We have previously shown that a humanized single chain antibody against IGF1R (IGF1R scFv-Fc) stimulated IGF-IR signaling *in vitro* when MCF-7 cells were treated with scFv-Fc for 10 minutes. The antibody phosphorylated IRS-1 and activated both the MAPK and PI3K pathways. It also stimulated the proliferation of MCF-7 cells *in vitro* and did not inhibit IGF-I's ability to activate IGF1R or stimulate MCF-7 cell growth. In contrast, scFv-Fc has been shown to partially inhibit xenograft growth of MCF-7 cells in athymic mice. In this study, we examined the mechanism by which scFv-Fc could inhibit cell proliferation. When subconfluent MCF-7 cells were serum starved for 24h and treated with 250 nM scFv-Fc for different time periods, scFv-Fc downregulated IGF1R levels after about 2h and the levels were greatly reduced after 24h. In contrast, treatment with IGF-I over the same time period did not affect IGF1R levels. To determine if scFv-Fc made MCF-7 cells refractory to further IGF-I effects, MCF-7 cells were pretreated with scFv-Fc for 24h to downregulate IGF1R and then stimulated with 5 nM IGF-I. 24h pretreatment of cells with scFv-Fc inhibited the ability of 5 nM IGF-I to phosphorylate IRS-1 and blocked subsequent MAPK and PI3K activation. In contrast, cells treated with 5 nM IGF-I for 24h still retained the ability to be further stimulated by subsequent treatment with additional IGF-I. Moreover, pretreatment of MCF-7 cells with scFv-Fc rendered them refractory to further proliferation induced by additional 5 nM IGF-I as measured by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay. Thus, while scFv-Fc initially stimulated the growth of MCF-7 cells, it inhibited further growth effects of IGF-I by downregulating IGF1R. Our data suggest that pretreatment with scFv-Fc results in receptor downregulation and this in turn makes MCF-7 cells refractory to further IGF-I exposure. These results indicate that such chimeric single chain antibodies against IGF1R have future potential in breast cancer therapy by causing downregulation of receptor.

Figure 2: Silver stained image of two-dimensional electrophoresis of MCF-7 cells treated with IGF-I. MCF-7 cells were treated with 5 nM IGF-I for 10 minutes. Cells were lysed in buffer containing 8 M urea, 2 M thiourea, 4 % CHAPS, and 40 mM Tris base. 18 cm IPG strip with a pH gradient of 3-10 was rehydrated in rehydration buffer containing 8 M urea, 2 M thiourea, 2 % CHAPS, 0.5 % IPG buffer pH 3-10, and bromophenol blue. 60 µg of MCF-7 cell lysate was subjected to 2DE on 8-16% gel. Protein spots were visualized by silver staining using the GelCode silver stain kit (Pierce, Rockford, IL). Molecular weight markers are shown on the right.

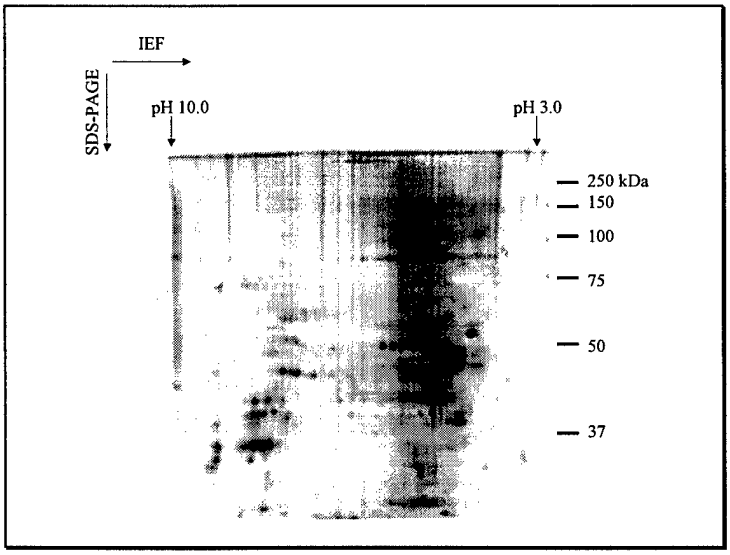

Figure 3: Two-dimensional electrophoresis of insulin receptor substrate-1 (IRS-1). MCF-7 cells were treated with 5 nM IGF-I for 10 minutes. Cells were lysed in buffer containing 8 M urea, 2 M thiourea, 4 % CHAPS, and 40 mM Tris base. 13 cm IPG strip with a pH gradient of 3-10 were rehydrated in rehydration buffer containing 8 M urea, 2 M thiourea, 2 % CHAPS, 0.5 % IPG buffer pH 3-10, and bromophenol blue. 100 μg of cell lysate was subjected to 2DE on the IPG strip and 8-16% acrylamide gel. 40 μg of lysate was applied to a sample application paper to the left of the IPG strip and subjected to only SDS-PAGE. Proteins were transferred to nitrocellulose and immunoblotted for IRS-1. Molecular weight markers are shown on the right.

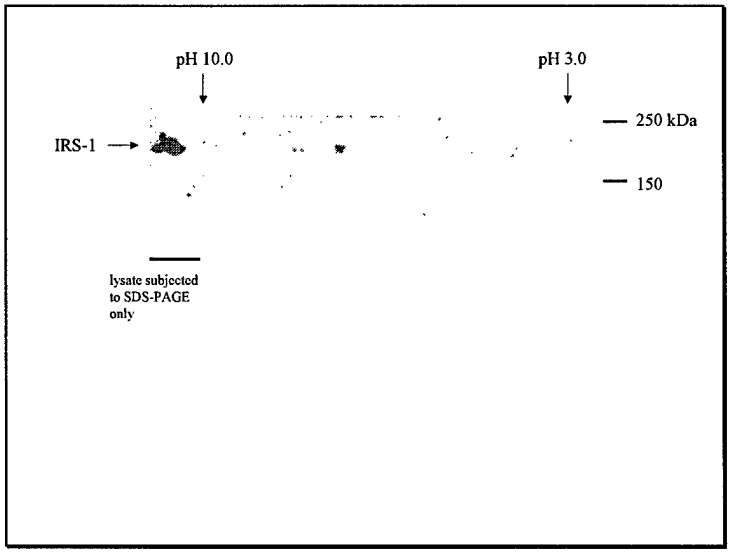

Figure 4: Two-dimensional electrophoresis of type I IGF receptor (IGF1R). MCF-7 cells were untreated (4 A) or treated with 5 nM IGF-I (4 B) for 10 minutes. Cells were lysed in buffer containing 8 M urea, 2 M thiourea, 4 % CHAPS, and 40 mM Tris base. 13 cm IPG strips with a pH gradient of 3-10 were rehydrated in rehydration buffer containing 8 M urea, 2 M thiourea, 2 % CHAPS, 0.5 % IPG buffer pH 3-10, and bromophenol blue. 100 μg of each cell lysate was subjected to 2DE on the IPG strip and 8-16% acrylamide gel. In 4 B, 40 μg of lysate was applied to a sample application paper to the right of the IPG strip and subjected to only SDS-PAGE. Proteins were transferred to nitrocellulose and immunoblotted for IGF1R using a polyclonal antibody against the β-subunit of IGF1R. The 97 kDa β-subunit of IGF1R is shown by the arrow.